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On the demonstration of defined, reversible associates ("reversible polymers") of acridine orange by means of absorption and fluorescence measurements in aqueous solution.

by Valentin Zanker.

Translated from Zeitschrift f. Physikalische Chemie 199-201: 225-258 (1952) by the Technical Library, Technical Information Division.

The vital pigment acridine orange is quantitatively analyzed in aqueous solution for its spectroscopic action in absorption and fluorescence in the entire range of concentration and pH. It is established during the sweeping of the pH scale that the pigment exists in 4 different forms which vary considerably both with respect to their proper color and the color of the fluorescent light. All dissociation constants are determined at 20° and applied to $K_{base} \rightleftharpoons ion^{+} = 3.55 \cdot 10^{-11}$; $K_{12ow} \rightleftharpoons ion^{++} = 4 \cdot 10^{-1}$ AND $K_{ion^{++}} \rightleftharpoons ion^{+++} = 1.4 \cdot 10^{2}$. As already discovered by Strugger, the form existing in the neutral range is the positive monovalent pigment cation which is extraordinarily dependent on the concentration in its optical behavior. The phenomenon of red and green fluorescence microscopy, is also analyzed quantitatively and reveals that the acridine orange cation forms the defined reversible associates that partly coincide with Scheibe's polymers and which permit analogies with those pigments that associate to double ions up to medium pigment concentrations (10^{-3}) molar). The thermodynamic magnitudes of the dissociation constants, free dimerization energy, heat toning and entropy important for the formation of dimers, are computed from the equilibrial state depending on the concentration and temperature, based on the data of optical measurement.

Even if no positive data are available on the numerical linkage of the higher associates, it may nevertheless be stated with certainty that the type of Scheibe's high polymer is not involved. The characteristic red fluorescent effect of concentrated pigment solutions is governed by an energy mechanism that may be assumed theoretically, but which has not been fully established by measurements carried out in aqueous solutions heretofore.

1. Statement of Problem

From the large number of acridine pigments utilized in staining techniques, acridine orange has moved into the foreground during the last decade, ever since it was recognized as a suitable stain and an excellent fluorochrome for vital

staining purposes in 1910 simultaneously by Strugger (2) and Rubaschow and

During initial cytophysiological studies with acridine orange, Strugger noted a remarkable affinity of this stain for the proteins of the protoplasts and recognized the possibility of staining plant and animal cells rapidly and carefully, permitting their examination in a stained condition. This further established that acridine orange staining may indicate different conditions of the plasma protein, demonstratable by fluorescence microscopy upon appropriate staining. Native (active) protein gives green fluorescence, denatured (dead) protein appears red. This differentiation was interpreted by Strugger as the concentration effect of the pigment upon electrostatic adsorption of the pigment cations in the cell plasma and the changes in the plasma protein in the living or dead cellular association were considered to be the cause of the dissimilar storage capacity. The lethal protoplasm stores a great deal of pigment and fluoresces copper red, while the living variety adsorbs relatively little and, for this reason, radiates a green fluorescence. Strugger was led to this assumption by subjective observation of the fluorescent light of aqueous pigment solutions, which show an advancing shift of the center of fluorescence from green via yellowish-green, yellow, yellow-orange, orange, ultimately to red in connection with constantly altered concentrations of 1 : 100,000 - 1 : 100. Strugger's observation and interpretation of fluorescence metachromasia as a concentration effect were unequivocally confirmed by Kolbel's (4) work with acridine orange pigment storage in living and dead cells.

The origin of this "concentration effect", i.e. the change in fluorescent colors in relation to the pigment concentration, had not been subjected to detailed study from the physical-chemical viewpoint and Strugger therefore suspected a correlation to the reversible polymerization of pseudoisocyanines discovered by Scheibe (3) at that time. As will be remembered, unstable changes in the

physical-chemical action were noted in this special class of pigments in aqueous solution upon exceeding of the marginal concentration tied to the temperature (at 20°C = $7 \cdot 10^{-3}$ mole/l), which were expressed primarily in the appearance of new, very narrow absorption bands, of resonance fluorescence and complete extinction of the dye solution. Since these sudden changes in the properties of pseudo-isocyanines are reversible with arbitrary frequency by means of temperature elevation or dilution, Strugger's assumption was quite credible after his visual observation of acridine orange had also established a reversible behavior in fluorescence. It was the aim of the present study to reexamine the physical-chemical action of ACRIDINE ORANGE ~~the pigment~~ in aqueous solution WAS REEXAMINED IN AN EFFORT ~~and~~ to discover the origin OF FLUORESCENCE of the "concentration effect" and, with it, of the metachromatic fluorescent effect, particularly from the correlation between absorption and emission based on the concentration.

The possibility of an extensive insight into the action of this stain seemed to be given by extending the study beyond the biological range of pH, i.e. in strongly acid and weakly alkaline solution, as well as into the area of the ultraviolet spectrum.

2. Purification of the Pigment and Preparation of Solutions

Earlier studies (6) had utilized acridine orange furnished by the Strugger Institute; the dye was later procured in a "standardized" form. The commercial preparations usually are in the form of zinc chloride double salt which is only partially soluable in organic solvents and contains numerous impurities. The stain was therefore subjected to the following purification for the purpose of optical studies:

After solution of the double salt in alcohol, the dissolved portion was filtered off from insoluble residues, strongly diluted with water and the yellow dye base was precipitated with diluted NaOH solution. Following rapid filtration and careful desiccation, the base was dissolved in $CHCl_3$ and purified chromatographically via Al_2O_3. The adsorbed dye was subsequently extracted with $CHCl_3$ and the solution was compressed until crystallized. The precipitating molecular compound contains 1 mole $CHCl_3$ which is easily separated during vacuum desiccation. The solvent-free product melts at 180° - 181° under normal conditions (melting point of the free base according to Beilstein 22, 487 = 180-181° or 181-182°).

The desired dye salts were obtained in a very pure state by solution of the pure base in alcohol and addition of a corresponding amount of mineral acid, followed by precipitation with ether. All studies under discussion were conducted with easily soluble dye chloride.

The preparation of buffer solutions were based on the "universal buffer" consisting of citrate and phosphate mixtures as listed by MacIlvaine (7), covering the biologically significant range of pH 2 - 8. Alkaline solutions were prepared as glycocoll - NaOH mixtures according to Kordatzky (8). The acid solutions consisted of H_2SO_4 - water mixtures up to the concentration of 78% sulfuric acid.

3. Measurements of Light absorption relative to concentration, PH and temperature.

a. Apparatus: Measurements in the visible spectral range were conducted by the photoelectric deflection method with a selenium photoelectric cell as radioaction receptor. Using the high intensity Leitz-Monochromator coupled to a 30 W Wolfram helical lamp. The range of 4,200 - 6,000 AU could be scanned with a median spectral width of the measuring light amounting to 40 - 50 AU

The relatively small energy fraction of the light source as well as the loss in sensitivity on the part of the selenium photoelectric cell in short wave blue require amplification to 60 - 70 AU in this range, but even then, absorption measurements with an absolute error of maximally $\pm$ 2% are still possible due to the great half width of the molecular bands (1,000 - 1,500 cm^{-1}). This fact has been confirmed repeatedly through photographic measurements with a spectral width of 1 - 3 AU.

The ultraviolet spectral range was measured predominantly according to the measuring principle of items (9) "comparison spectra" arranged after Holbam. Kortum and Szigetts (10), using an H_2 tube as light source. Utilizing the large quartz spectrograph of Fuess. the spectral width amounted to 0.3 AU in the short wave ultraviolet and 1.0 - 1.2 AU on the margin of the visible range. Subsequent controls and measurements were also carried out with the photoelectric UNICAM quertz spectrophotometer SP 500 which is eminently suited for the reception of quantitative absorption curves in the ultraviolet and visible areas. The average spectral width of the measuring light in this method is 4 - 7 AU in the ultraviolet spectral range.

The instrument depicted schemetically in Figure 1 was designed for measurements of light absorption relative to the temperature. The cuvettes with the solutions are fastened to a stable Al stop plate on a vertical carrier, equipped with 2 perforations with sleeves. These convey the circulating fluid which is maintained at a certain temperature by the thermostat. The cover plate with the cuvette support is tightly connected with the Al cast-metal housing. The latter having a surface-ground lip. Plane parallel glass or quertz panes are inserted back and front, permitting the transmission of the arrangement along the optical axis. The whole system is placed on an

optical runner and may be moved laterally between guide stops. This permits exact local reproduction of both cuvettes for optical measurement. The device is suited primarily to temperature ranges in which the utilized solvent does not as yet possess an excessively high vapor pressure and where the viscosity of the thermostat fluid is relatively low.

b. Measuring Results:

1. Relative to the concentration: Figure 2 reflects the results of absorption measurements related to pigment concentration from saturation to 10^{-6} molar solution. All measurements were conducted at a constant temperature of 20°C and pH 6.0 of the citrate-phosphate buffer. The absolute salt concentration amounted to 19 g/l. pH 6.0 was chosen for two reasons: Firstly, because the acridine orage base is already entirely transformed to the dye salt at this value - which will be proved later - and secondly, because vital staining is conducted exclusively with neutral solutions.

Figure 2 shows as do all subsequent absorption curves, the dependence of the molar decimal extriction coefficient upon the wave number. The curve progressions of the various concentrations clearly indicate that acridine orange is governed by a concentration - tied equilibrium whose greatest variability is shown in the concentration range 10^{-3} - 10^{-5} molar. Beer's law therefore, is not fulfilled in the entire concentration range tested. Asymptotically derived marginal values are obtained only upon approaching saturation or at dilutions $< 10^{-6}$, indicating the law's validity. The maximum of the characteristic long wave bands becomes static in high dilutions connected with a constant wave number of 20,400 cm^{-1} and reaches a limit (determined by extrapolation) at a K value of nearly 61,000 and a concentration

of 10^{-7} - 10^{-8} molar, while the short wave secondary band at these dilutions is indicated only by a slight shoulder. The secondary band proper becomes visible only starting with concentrations > $5 \cdot 10^{-5}$ molar; it experiences an inial short wave shift by normal quantities from its position in the concentration range $5 \cdot 10^{-5}$ - $1 \cdot 10^{-3}$ molar, and, starting with C = 10^{-3} molar, quickly reaches a terminal value in the neighborhood of 22,150 cm^{-1} and a K value of 29,000.

Acridine orange thus reveals a behavior that has been observed in connection with numerous pigments and which has been studied in a similar fashion by Rabinowitsch and Epstein (11) with thionine and methylene blue (MB)$^{+}$. In this case the authors considered the long wave primary band as belonging to a monomericion and the short wave secondary band as belonging to a dimeric ion, and determined the K_{max} values of both bands by extrapolation of the experimentally derived K values to very small (C=0) and very large (C=∞) pigment concentrations. Under the assumption of double ion formation $MB^{+} + MB^{+} \rightleftharpoons MB_2^{++}$, a comparison was undertaken between computed and observed K values, leading to confirmation of the dimerization hypothesis.

As in the case of the pigments thionine and methylene blue, such an equilibrium of $AO^{+} + AO^{+} \rightleftharpoons AO_2^{++}$ may also be assumed for acridine orange $(AO)^{+}$ up to concentrations < 10^{-3} molar, but more concentrated solutions must be excluded from these considerations, since increasing band shifts and displacements of intensection can only be understood with the assumption of the formation of higher associates. As shown by a later study, the short wave band characteristic for a double molecule would have a center of gravity [illegible] cm^{-1}. This value, however, [illegible]

toward higher wave numbers up to saturation. The absorption curves of solutions $< 10^{-5}$ molar clearly reveal that this short wave band (center of gravity at 21,500 - 21,600 cm^{-1}) is implied as a shoulder even at very high dilutions. Previous measurements of alcoholic solutions (12) also show this short wave shoulder, although no signs of association are present and Beer's law is fulfilled along a wide concentration range. Moreover, since the determination of the absolute height of the dimeric band in aqueous solution is vague due to the superimposition of the long wave primary band, this method was not utilized for the demonstration of dimeric formation. As Scheibe (13) and Ecker (14) already showed in connection with pseudo-isocyanine, the formation of double ions may be deduced with certainty only fromthe falling function of the monomeric band.

When an unknown number x of monomeric acridine orange cations AO^{+} combines to a polymer P, the law of mass action is:

$$\frac{(C_{AO^{+}})^x}{Cp} = K$$

where $C_{AO^{+}}$ is the monomeric and Cp the polymeric concentration. The initial concentration is then composed of $C_o = x \cdot Cp + C_{AO^{+}}$. In the case of acridine orange, the primary band located at 20,400 cm^{-1} must be considered as the monomeric band which permits the determination of the monomeric concentration and the quality $x \cdot Cp = C_O - C_{AO^{+}}$ from the concentration factor of its K_{max} value, as described in more detailed below. When x is assumed to be a constant in a certain concentration range, the logarithemics of these two values are entered graphically, the curve drawn in Fig. 3 is obtained. The value for x = 2.0 may be derived from the inclination of the curve, which represents a virtual line in its medium

ca. mat. [illegible] the formation of dimers in the first phase of association is positively confirmed for acridine orange. Higher associates are very probable as saturation is approached, but the proof of more numerous linkages cannot be offered from absorption spectra due to the indetermination of position and height of the superimposed short wave bands.

The circumstance of monomeric band dependence on the concentration was used further in the determination of the dissociation constant K_D and free dissociation energy ΔF. All computations in the concentration range in question were based on exclusive dimer formation, subjecting the dissociation equilibrium to the following conditions:

$$AO_2^{++} \rightleftharpoons AO^+ + AO^+ = K_{D_{20}} \quad \text{or} \quad K_{D_{20}} = \frac{(AO_2)^{++}}{(AO)^+ (AO)^+}$$

When the fraction of monomers is designated a and that of dimers the equation changes to:

$$K_{D_{20}} = \frac{1}{C} \cdot \frac{1-a}{2 \cdot a^2}$$

where C is the total concentration of dye, i.e. $C = AO^+ + 2\,AO_2^{++}$. The extinction coefficient K, which determines the concentration of monomers, is established with due regard for short wave superimposition, and is applied to a maximal value (100% monomeric ion) of the monomeric band at 20,400 cm^{-1}. This K_{max} value lies at K = 61,000, as already stated. The same relationship applies to the ultimate magnitude of the K value of the monomolecular band upon the approach of saturation concentration, in whose range the existence of monomeric ions is improbable. By extrapolation and determination of the overlapping portion of the short wave band, this terminal value can be established at K ~ 9,000.

The following K_{D20} values were derived by this method, and the corresponding F values were obtained from the relation [4] $F = RT \cdot \ln K^{comp} =$

C	C · α	C · (1−α)/2	K_{D20}	ΔF
1 " -3	1.32 · 10⁻⁴	4.37 · 10⁻⁴	3.0 " 4	5.1 " 0.1
5 " -4	0.94 · 10⁻⁴	2.07 · 10⁻⁴	2.3 " 4	- " -
1 " -4	3.50 · 10⁻⁵	3.25 · 10⁻⁵	2.6 " 4	- " -
5 " -5	2.33 · 10⁻⁵	4.31 · 10⁻⁵	2.3 " 4	5.7 " 0.1
1 " -5	7.57 · 10⁻⁶	4.22 · 10⁻⁵	2.3 " 4	5.7 " 0.1
5 " -6	11.24 · 10⁻⁶	2.15 · 10⁻⁵	2.3 " 4	5.7 " 0.1

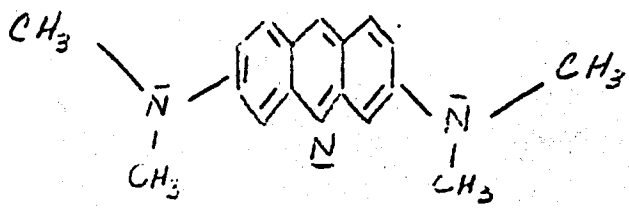
The median dissociation constant K_{D20} may be established at $2.2 \cdot 10^4$ l/mol from the last three values, the free dimerization energy F at 5.7 K cal/mol. The increase in the constant with higher concentrations can be explained only by the assumption that the dimeric concentration included in the equation is too great, i.e. that equilibria of higher associates already asser themselves in the concentration range $> 5 \cdot 10^{-5}$ molar.

II. pH dependence: The curves in Fig 4 and 6 show the dependence of the absorptional process on the pH of the solution in the visible and ultraviolet spectral range. The five measurements in the neutral and weakly alkaline range represented in Fig 4 permitted precise determination of the K_{D20} value and the electrolytic dissociation constant. In Fig 5, these results have been evaluated for a concentration of 10^{-5} molar.

As evident from Fig 4 both types of molecule possess a constant, ultimate K value of the preceding or following form at their characteristic maximum of 20,400 and 23,000 cm^{-1}, respectively. The evaluable extinction coefficient of one molecular form therefore had to be found from the difference between the reading and the concentration - dependent portion of the othermolecular form. It was assumed in this connection

10^{-6} molar dye solutions which had yielded a constant of $K_1 = 2.4 \cdot 10^{-11}$ (P_K value = 10.60) upon similar evaluation. A change in this value upon further dilution is improbable, since the dimeric concentration at 10^{-6} molar amounts to only 2 - 3%.

The yellow form with the first band maximum at 23,000 cm^{-1} which alone exists in the strongly alkaline range starting with pH 12, is the color base, i.e. The electrically neutral pigment molecular of the following formulation.



Acridine orange base.

The solubility of the base in water is very poor due to its hydrophobic character, so that 10^{-5} molar solutions are just able to exist in molecular dispersion and therefore may be included in comparative series of measurements. Cataphonetic studies of weakly alkaline dye solutions in a continuous-current field have confirmed the electroneutral nature of this molecular type (15).

From the weakly alkaline to the neutral range, the first H^+ ion is added to the neutral molecule, whose charging is practically terminated at pH6. The positive, monovalent dye cation which was examined in the preceding chapter for its concentration dependence is now formed, being approximately described by the indicated mesomeric forms.

that changes in pH would not alter the relative curvature of the individuals due to a drop or rise in concentration, and that the curves at pH 5.0 and 12.0 are characteristic for the two types of molecule. The first transition range of the dye from yellow to orange was established according to the method with the equilibrium of its two forms (P_K value) at pH = 10.45 and the dissociation constant $K_1 = 3.55 \cdot 10^{-11}$. The fact that a genuine equilibrium exists here may be recognized by the common intersection of all curves in the pH range 1.2 - 12 at 22,450 cm^{-1}.

Since previous absorption measurements had failed to confirm the validity of Beer's law for 10^{-5} molar solutions, and the determination of dissociation constants had initially been conducted at this concentration due to technical considerations. An influence of the equilibrium of H^+ association (prototropic equilibrium) owing to the continued supply of monomeric ions from the dimeric equilibrium had to be assumed, and with it, a small error in the determined dissociation constant.

Combination of the two equilibria

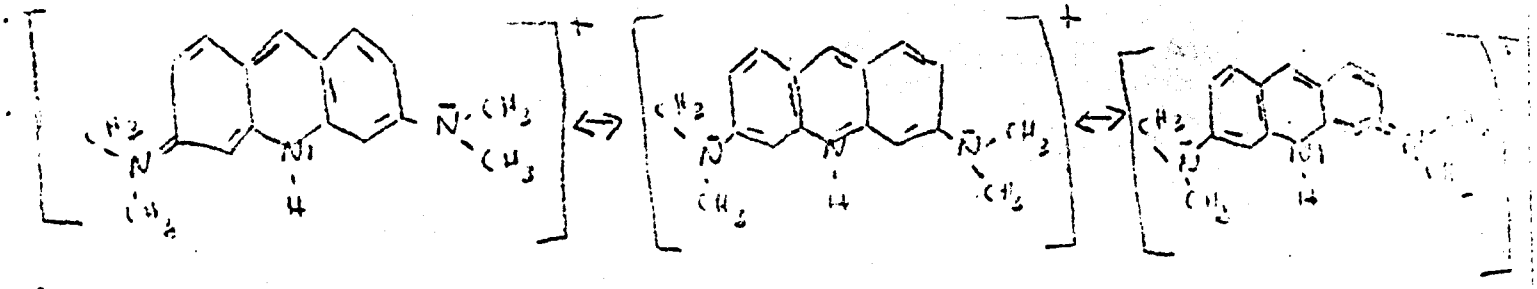
$$\frac{(AO_B) \cdot (H)^+}{(AO_j)^+} = K_j \text{ and } \frac{(AO_2)^{++}}{(AO)^+ \cdot (AO)^+} = K_D$$

$$\text{Yields } (AO)^+ = \sqrt{\frac{(AO_2)^{++}}{K_D}} = \frac{(AO_B) \cdot (H)^+}{K_j}$$

$$\text{and further, } \frac{K_j}{\sqrt{K_D}} = \frac{(AO_B) \cdot (H)^+}{\sqrt{(AO_2)^{++}}}$$

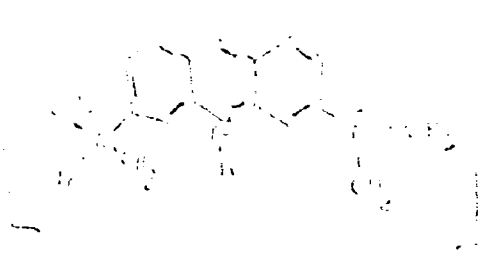
where AO_B and AO_j are the concentrations of the acridine orange base and the cation AO^+, respectively, and K_j and K_D are the acid and dimeric dissociation constants, respectively.

By insertion of the values $K_D = 2.2 \cdot 10^4$ and $(AO_2)^{++} = 1.22 \cdot 10^{-6}$ for concentration $C = 10^{-5}$ molar. The precise value of $K_1 = 2.37 \cdot 10^{-11}$ ($P_K = 10.62$) may be computed. This quantity agrees superbly with the results of control measurements of pH - dependence of the extriction coefficient in



Acridine Orange cation

When the first H⁺ ion is attached to the ring N atom, a long resonance chain results for the reciprocal action with the radiation field, in which all N atoms that qualify as charge carriers, participate. Since proton attachment at this point is connected with an enlargement of the transitional moment and promotion of the reciprocal action with the radiation field, vigorous long wave absorption bands may be expected, which indeed are observable in the spectrum. If, however, one of the two substituent N atoms is charged first, then one auxoderomic groups could be lost to resonance with the remaining JL electron system and the absorption picture would resemble that of electroneutral 3- or 6- aminoacridine. Measurements carried out by Craig and Short (16) and Turnbull (17) with monoaminoacridines, The results of which are partially entered in a separate coordinate system in Fig. 6 for 3- aminoacridine, negate.the second possibility. The above-mentioned authors studied similar problems in connection with mono-substituted acridines and also found that the ring nitrogen is more basic than the substituent N atom.



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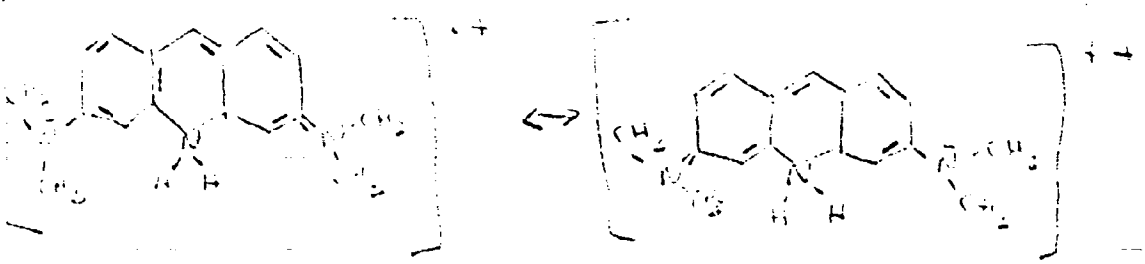

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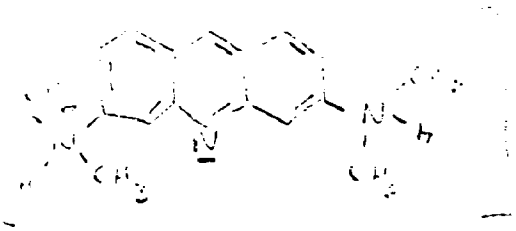


III

As further shown by the curves in Figs. 4 and 5, the K value of the characteristic band remains constant from pH 6 to ca. pH 1.5, i.e. The positively monovalent dye cation is the only existing type of ion. The commencing drop of the "orange" dye band and the simultaneous increase in extinction in the long wave spectral range, shows the beginning second transition range of the pigment from orange toward red. Again there is an isosbestic point at 19,550 cm^{-1} (K value 16,000) as a characteristic for an equilibrium between two forms of dye, of which the red form existing in the acid range can only be twice ionogenic. The acidity range between 0.1 normal and 20% H_2SO_4 has been subjected to additional measurements, which confirmed this intersection. The equilibrium of the orange and red molecular forms in this case was determined from the pH dependence of the two ultraviolet absorption bands at 33,300 and 37,200 cm^{-1}. In the case of ca. In H_2SO_4, a P_K value of 0.4 was obtained, corresponding to a dissociation constant of $K_2 = 4 \cdot 10^{-1}$.

Of the possible formulations that consider only the N Atom as charge carriers, the three essential ones are depicted below. Formula III is immediately eliminated, since in this case both auxochromes would be prevented from color combination and the spectrum of free acridine would present. This is not the case, however, as shown by the spectroscopic findings in Fig. 6. Of the remaining formulas I and II, the structure of I seems to be the more stable, since in the case of II the ring N atom would be eliminated from the resonance of the remaining π electrons and the rigidity of the molecule would be considerably loosened at this point.

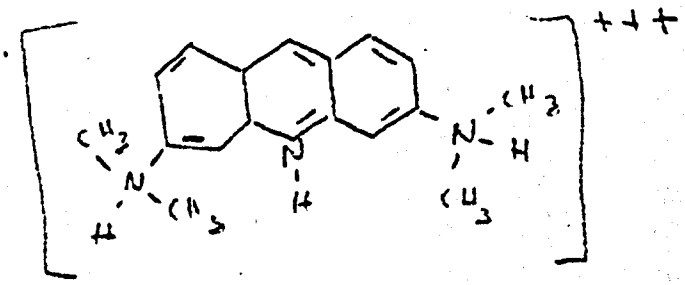
Formula I so also supported by the spectrum of 3-aminoacridine cation based on Turnbull's measurements, who in this case proved the charge of the ring N atom and the freedom of the auxochromic amino group. A similar "optical" molecule is present also in structure I, since one of the substitutes is no longer effective optically due to the charge. In a comparison of the appropriate absorption curves in Fig 6, this similarity is easily recognized from the form and position of the depicted long wave bands. On the other hand, formula II is substantiated by the molecule's symmetry which, together with the extended resonance path via methine carbon, explains absorption of longer waves. The instability of the dye solutions in the range of 5 - 20% H_2SO_4 also points in this direction since the molecule in an activated state is rotated slightly from the plane position due to the loosening of the ring N atom linkage, and the band itself is placed under great stress thereby. After storage of these solutions of medium acidity for several months, an irreversible degradation of the dye was noted repeatedly, whereas highly acid soltuions revealed an unusual stability. Similar observations of instability with spectral changes were made also in connection with the green form of crystal violet, which customarily is marked by extraordinary instability. Förster (18) offered a theoretical explanation of the optical behavior of this dye belonging to the triphenyl-methane series. He interpreted the energy cleaverage during the transition to malachite green or to the green form of crystal violet as a neutralization of 3-fold energy degeneration. A similar case of degeneration exists in the positively monovalent acridine orange cation. When this degeneration is suppressed by charging one of the

substituent N atoms, an energy cleavage ought to result which indeed is demonstrable in the spectrum in a form similar to that of crystal violet. Thus, formula I seems to be secured, especially since now the long wave absorption and, possibly, the instability become clear.

The condition upon further increase in acidity are again evident from Fig. 4 and, finally, from Fig. 6. In the concentration range of 20 - 60% H_2SO_4 the characteristic band group in the visible is continuously decreased in its integral absorption, making this color change the 3rd transition range of the dye. Red coloration is completely absent in the 73% H_2SO_4 solution and a weakly yellow hue that persists up to 96% H_2SO_4, indicates the presence of a new 3 times positive molecule. In a series of measurements, similar to the preceding equilibrium ion f^1 ⇌ ion f^2 this third equilibrial constant ion f^2 ⇌ ion f^3 was also derived from the change in K_{max} of the two characteristic bands at 38,700 cm^{-1} and 41,700 cm^{-1} in relation to the H_2SO_4 concentration and established at 51.5% H_2SO_4 to be $K_3 = 1.4 \cdot 10^3$. The pH values of the H_2SO_4 - water mixtures have been extracted from the acidity - pH curve constructed by Hammett (19) and Schwarzenbach (20).

Fig. 6 contains the spectroscopic proof of the fact that a molecular type of the indicated constitution is involved in the highly acid pH range in which both auxochromic N atoms are neutralized by treatment of the resonance with the aromatic JL complex. The resulting chromophone therefore must be identical with that of the acridicium ion. This is actually the case, as shown by the absorption picture in Fig. 6. An additional proof is the extraordinarily characteristic ice-blue fluorescence, whose bands coincide in position and height with those of the acridinium ion, corresponding to the differences in absorption. The nominal short and long wave displacement

of both activated states is insignificant and is probably caused by a weak linkage of the charged auxochromes. Numerous studies of various authors (21) with acid solutions of aniline, pyridine, dinoline, isodinoline, acridine and anthracene also offered spectroscopic proofs of the identity of ring N and C atoms in aromates, as well as of the ineffectuality of the NH_2 group in "salt formation."



Acridine orange cation[III]

Recent raman-spectroscopic and cysoscopic measurements (22) of concentrated H_2SO_4 solutions have supported the assumption that autodissociation of H_2SO_4 to $H_3SO_4^+$ and HSO_4^- produces a highly acid cation which, to all appearances, must be held responsible for high acidity and, consequently, for the electromotive potential. Schwarzenbach had already suspected the existance of a sulfuracidium ion $H_3SO_4^+$ in his compilation of normal acidity potentials and the "superacid" acidity curve.

III. Temperature dependence: Fig 7 shows measurements of temperature dependence of light absorption in the visible range. Since the properties of the dye base forming in the alkaline range and the bivalent and tribalent ions existing in the highly acid pH range were of no further interest, the effect of temperature was examined only at a median, neutral pH level.

It was shown by the choice of a favorable median dye concentration that a temperature change of about 90° causes the same optical changes as those effected by an increase or decrease in concentration by almost two decimal powers.

After measurements of concentration dependence in this range had essentially established a dimeric equilibrium, the dissociation constant K_D could be computed as a function of the temperature from the well-known relation $K_D = \frac{1}{C} \cdot \frac{1-a}{2\,a^2}$. The monoberic concentration was again determined from the marginal values indicated in Part K, under consideration of band overlapping. The relation

$$\frac{d \ln K}{dT} = \frac{\Delta Q}{RT^2} \quad \text{or} \quad \Delta Q = RT^2 \cdot \frac{d \ln K}{dT}$$

Yielded the dimerization heat ΔQ and the equation $\Delta S = (\Delta Q - \Delta F)/T$ produced the dimerization entropy. The results are compiled in the following table:

°C	Dissociation constant $K_D(E)\,T$	Free energy of dimerization ΔF in kcal	Heat of dimerization ΔQ in kcal	$\Delta Q - \Delta F$ kcal/mole	ΔS cal/mole
6	6.0×10^{-4}	6.1	8.4	2.3	7.3
17	2.3×10^{-4}	6.0	8.3	2.3	7.9
26	1.5×10^{-4}	5.9	8.3	2.4	7.7
52	7.5×10^{-5}	5.9	8.3	2.5	7.7
73	3.5×10^{-5}	5.6	8.4	2.9	7.1
99	1.7×10^{-5}	[illegible]	8.5	3.1	7.4

The computed values in the table allow the determination of the dimerization heat ΔQ at an average of 8.4 kcal/mol and dimerization entropy ΔS at 8.0 cal/mol. Since the value of the dissociation constant K_D at low temperature probably too high owing to exclusive consideration of dimeric formation, this should explain the nominal rise in entropy with falling temperatures. As mentioned in Part K, the increase in the K_D value with the concentration again should be ascribed to the formation of higher associates.

4. Measurements of the Energy Distribution of the Fluorescence in Relation to the Dye Concentration

a) Apparatus: An Osram-Hg-maximum pressure lamp HBO 200 was used as exciter light source; its visible radiation was completely filtered with a Schott UG 11 filter and a saturated $CuSO_4$ solution of 2-3 cm thickness. The ultraviolet exciter radiation is then composed of a weaker continuence of 3,300 - 3,900 AU with strongly expanded lines superimposed at 365 and 366 mu. In order to prevent reabsorption as much as possible, work was carried out in incident energising light and at a universal stratal thickness of the dye solution. Exposures were made with the small Fuess glass-spectrograph, aperture ratio 1:4. A slit 0.025 mm in width produced fluorescent light with a spectral width of 2-5 AU in the 5,300 - 7,000 AU range.

Most of the plates utilized were of the type Agfa spectral-red-rapid. Spectral total and infrared 700 and 750 were used in the determination of long wave drop in fluorescence. In spite of storage for several years,

spectral-red-rapid had undergone only an insignificant change in sensitivity compared to new emulsions; considerable loss in sensitivity was shown by spectral-total-hard, especially in the long wave red.

Neuhaus's (23) standard method for the exposure of spectrum fluorescence spectra was modified for material reasons to the extent that the conversion of density curves to energy distribution curves made use of sensitivity curves obtained by means of a Pt-6 graduated filter and an emitter of known color temperature and energy distribution. The determination of the sensitivity curve was carried out in such a manner that, initially, the density was established for the appropriate spectral range in intervals of 50 to 50 AU, depending on the relative energy (found with the Pt graduated filter), utilising the comparison emitter. The characteristic film curves now enable us to find the corresponding relative values for different absolute density values (S = 0.10; 0.25; 0.35; 0.50; 0.75 and 1.00) and to multiply the former with the absolute value of the emitter. The minimal value of absolute energy belonging to a certain wave length was then equated with 1 for the appropriate phase of density and the other values were then translated accordingly.

Fig. 8 shows a number of such sensitivity curves for stored spectral-red-rapid, applied to an equienergic spectrum. The various degrees of density are determined by the variable graduation of their wave length dependence, while maxima and minima are induced by the spectral sensitivity of the sensitized emulsion. The absolute maximum of sensitivity in this type of plate was found at 6,500 AU for all degrees of density, causing all curves to meet in this point.

These curves permit a rapid conversion of density values to relative energy values and, further, determination of the energy distribution of fluorescence from the measured density. In controls of utmost precision, the graduated comparison emitter may be added at the start of the exposure series, permitting the recheck of sensitivity curves. In the case of intermediate values, an appropriate interpolation is made between the upper and the lower levels of density. The exposure time for the exposure of fluorescent radiation may be matched with that of the W emitter by means of the high-intensity HBO 200 lamp.

b) Results of measurements: Fig 9 shows the results of fluorescence measurements in relation to the dye concentration. As is evident from the graph, these tests were applied only to biologically interesting conditions of fluorochroming in a neutral medium, i.e., to the range of the monovalent dye cation. Highly acid or alkaline solutions are irrelevant in this connection, since they cannot be used for vital staining. In order to make relative comparisons possible, all curves were applied to the fluorescent intensity and the latter was equated with 1. As in the representation of Fig 2, which shows the behavior in absorption along the entire concentration range, the determinations of relative fluorescence distribution made use of the same concentration interval, permitting precise observation of energic relations derived from optical properties.

Fig 9 shows that there are 2 essential band centers in fluorescence, resulting [illegible] certain similarity in the absorptive behavior. One has its maximum at 20,[illegible] cm^{-1} in the [illegible], the other at 15,[illegible] cm^{-1} in the long wave red. As in Fig 2, one marginal zone of the fluorescence band is reached only at [illegible] of [illegible] [illegible] band [illegible] [illegible] [illegible] values of [illegible]. The [illegible] is [illegible] interval, in [illegible] band

is suppressed or magnified relative to the other. In this medium concentration range, the visible color of fluorescence changes continuously from green via greenish-yellow, yellow, yellowish-orange, orange, reddish orange to red, whereas objective spectral observation discloses distinct energy levels that are concentration dependent in their relativity. The interval between the green and red band centers amounts to 18,750 - 15,250 = 3,500 cm^{-1}. This corresponds to an energy difference of almost exactly 10 kcal. In absorption the extreme band maxima are located at 20,400 and 22,150 cm^{-1}; this yields a difference of 1,750 cm^{-1} = 5.0 kcal. Upon the fixing of a symmetrical line (19,600 cm^{-1}) and an analysis of the curves in absorpiton and fluorescence, Lewschin's mirror symmetry law is confirmed up to a concentration of $1 \cdot 10^{-5}$ molar, but the strongly widened absorption band at 22,150 cm^{-1} seems to owe its inception to the superimposition of two bands at ca. 21,500 - 21,600 cm^{-1} and 22,800 - 23,000 cm^{-1}, of which the one with the longer waves does not possess the corresponding radiation probability in emission as indicated by the transition in absorption. The law os symmetry is still not confirmed for the red fluorescent band and can be considered fulfilled only when absorption bands with even shorter waves (at 24,000 cm^{-1}) are present. As may be concluded from the spectroscopic course of the absorption curves towards higher wave numbers, such absorption bands of low intensity may be assumed to exist. The drop in intensity of the red fluorescent band observed, for the time being, only qualitatively, indicated a considerable difference in the two bands' intensity when compared to the very vigorous green fluorescence and thus conforms to the weak bands suspected on the short wave side and the characteristic long wave absorption band. The connecting links between the bands drop in absorption (determined by shelling) from the long wave side to the short wave side, whereas the fluorescent bands, indicated by the levels

increase from short to long waves. [illegible] to indicate the existence of a mechanism that first prohibits oxidation after a certain dye concentration has been reached, and later allows [illegible].

The question concerning the cause of characteristic photochromic variations is therefore identical with the [illegible] into different dye levels situated between the basic ground form and the first ontogenic state, whose transition from the ground state to absorption is prohibited, i.e. allowed only to the extent shown by the asymptotic course of absorption on the long wave side.

Repeated absorption measurements carried out with great spatial thickness and high pigment concentrations failed to reveal signs of absorption bands in the questioned range from 25,000 to 15,000 cm^{-1} in aqueous solution. Fig 10 shows such a reading of residual absorption of a 10^{-2} molar, buffered dye solution (pH = 6.0). As evident, the long waves are followed by ultimate absorption which probably is caused in part by the scattering of the relatively viscuous dye solution.

5. Summary and Interpretation of Test Results

Empirical results of absorption measurements in relation to pH prove that the acridine orange molecule may exist in 4 different forms in the practically accessible range of pH. The alkaline range contains the relatively strong, yellow-colored dye base whose ring and substituent N atoms carry free electron pairs marked by a differentiated proton affivity. Toward neutral, the ring nitrogen is charged first, resulting in a positively monovalent cation of orange color which is alone present in the pH 6.5-1.5 range. Both types of molecule are coexistent in a [illegible] relationship in the first transitional

range of pH 6.5-11. The types are differentiable fluorescentially; the neutral molecule fluoresces with a center of gravity at 4,900 AU with a blue-green color, while the maximal fluorescence of the dye cation is located in the green at 5,350 AU starting at concentrations < [illegible] molar. These results are significant for biological vital staining and its correct interpretation. The colorimetric dissociation curve (P_K value about 10.2) constructed by Hilbel (4) in this connection was confirmed and conclusively established. Upon further amplification of acidity, a second H^+ ion is attached to one of the auxochromic groups, forming a doubly positive red dye cation. Finally, the substituent N atoms are completely charged, causing both auxochromes to be eliminated from resonance with the aromatic nucleus and revealing the spectrum of the substituent-free, weakly yellow acridine cation.

Studies of the properties of the biologically relevant dye cation revealed an effect of concentration and temperature on the measured absorption curves, as already known in part from various papers (25), especially those of Scheibl on pseudoisocyanines and that of Rabinowitsch and Epstein on similarly constituted methylene blue. Methylene blue shows properties that point to the formation of dimers in the tested concentration area, but investigations by Lewis, Goldschmid, Nagel and Bigeleisen (26) as well as Vickerstaff and Lemin (27) indicate the existence of even higher associations. The pigments examined by Scheibe showed an equilibrium between monomeric and dimeric dye ions along a wide range of concentrations, characterized by an isosbetic intersection. A rise in concentration induces an absorption band with shorter waves, ascribed to the formation of a loose, more highly polymeric band of the dye cation. The interesting polymeric form appears with the long wave, intense band only at very high pigment concentrations (> [illegible] molar)

The remaining bands disappear for the most part and the isobestic point again proves the consequential equilibria.

The acridine orange cation differs from a large number of heretofore closely examined dyes by the fact that the first short wave band which usually indicates the first step of a polymerization, i.e. the dimer, persists as a superimposed shoulder up to great dilutions and for this reason must be considered to be the oscillator of the monomer. Although increased concentrations produce the short wave maximum, the latter continuously undergoes displacement in position and height due to the constant change in intensity of the superimposed long and short wave bands, resulting also in changes in the curve intersection of the two magnitudes. It is only at concentrations $< 5 \cdot 10^{-5}$ molar that an approximately common intersection is recognized, indicating the predominance of monomeric and dimeric ions in these solutions. The continuous displacement of the intersection and the magnification of the dimeric dissociation constant K_D therefore point to increased overlapping of higher equilibria starting at concentrations of $> 5 \cdot 10^{-5}$ molar. An interpretation of the total behavior in absorption may be derived from the data only by assuming that formation of dimers is predominant up to concentrations of $< 5 \cdot 10^{-5}$ and that successively higher associates are formed up to saturation (probably out of the dimers), and that these are interconnected by reversible equilibria. These micelles of higher aggregate might possibly be building stones of the crystal.

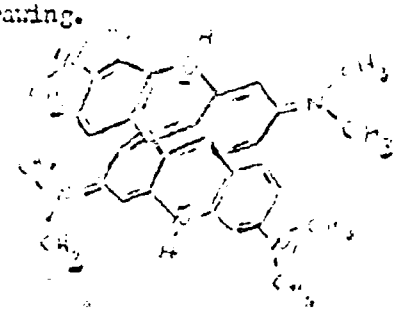
Measurements of molar conductivity and dynamic viscosity also point in the same direction, these, however, can be discussed only after procurement of extensive experimental material.

The reason for the concentration effect, i.e. the clumping of positive dye cations into dimeric and more highly associated ions, must be found in London's dispersion forces, already pointed out by Mortum (28) in his study of the absorption spectra of dye solutions. According to London (29), these forces are a sequel of mutually, short-lived disturbances in the molecular electron movements. According to Forster (31) they may be composed of the combined force of one electron from each molecule and effect an attraction between the participating molecules at an average rate. The more easily deformed outer electrons contribute most to this process. These attractive forces are particularly great in the typical dyes that possess strong absorption areas in the long wave owing to the great number of electrons with relatively weak binds. As a consequence of this attraction, dye ions group themselves into dimeric units and larger aggregates. Coulomb repulsive forces of equally charged ions in aqueous solution are considerably attenuated by the high dielectric constant of the solvent, whereas the attractile dispersion forces are not affected by the magnitude of the dielectric constants.

The prerequisites for the occurrence of strongly associating dispersion forces are present in the acridine orange cation, since an intense, long wave absorption band is observed initially due to the hard resonance between different mesomeric marginal forms and the charge resonance between ring N aton and substituent N atoms. The exclusive seat of the charge on the N atoms, the flat structure of the aromatic nuclear framework whose plane is not transcended by space-filling atoms, as well as the incorporation of anneloxanes in the flat molecular orientation, favor the effectiveness of the dispersion forces between the hydrophobic CH skeleton of the dye ions [illegible] the unusual association tendency of the acridine orange cation

in aqueous solution - - characterized by the computed thermodynamic quantities. Similar considerations are probably valid for vital staining of biological interest and the variable storage of pigment in viable and dead cells. However, in this connection the conditions of electrical charge and the structure of the staining agent play an essential role, as already pointed out by Förster (2) and Kolbel (4).

It seems indicated for the interpretation of the metachromatic behavior, to transfer to acridine orange the theoretical investigations carried out by Förster (31) in connection with thionine and methylene blue, since they led to an understanding of the manifestations of absorption and fluorescence in the case of these dyes. Accordingly, the double molecule of the acridine orange cation is assumed to have an arrangement of individual ions as reflected in the following drawing.



Acridine orange double ion //

For reasons of stability the aromatic nuclei will be superimposed and the ring N atoms will be arranged in opposition, if association is caused by dispersion forces. The direction of the electron oscillation of the the visible absorption band must be assumed to be parallel, since they coincide in these molecules with the longitudinal axis of the individual molecules. The additional symmetry results in 2 normal oscillations of different frequency whose plane is displaced equally or by 180°. Synchronous oscillation with

the resulting dipole moment is permitted in absorption and emission, while the indicated anti-symmetric oscillation has a conversion moment of zero and therefore is optically inactive. The frequency distribution due to linkage results in a frequency that is higher in the symmetrical oscillator than in the monomeric one, and a lower frequency than the latter in the anti-symmetrical oscillator. Since the latter form of oscillation is prohibited in absorption, the dimer can only be expected to yield a short wave band.

Whether this interpretation is valid also for the resulting exchange action cannot be derived directly from Fig. 2, since the short wave dimeric band is too strongly overlapped. Under the assumption of a symmetrical drop of the primary band (20,400 cm^{-1}) toward the short wave side, this band has been established earlier (32) by peeling and its position fixed at approximately 21,500 cm^{-1}. As already stated elsewhere in this paper, the band does not represent an exclusive characteristic of the dimer, since this oscillator also participates in the monomer and is merely reinforced upon dimer formation. On the corresponding long wave side, i.e. at 19,300 cm^{-1}, the asymptotically oriented absorption curves show only superimposed bands of very low intensity, essentially confirming the theory in absorption.

Light absorption initially energizes the double molecule in the active, symmetrical form of oscillation, from which it changes into the form with lower frequency without emission, activated by unsymmetrical nuclear oscillation (again, according to Forster). This process must transpire with extraordinary rapidity, since otherwise it could not counteract the emissions from the higher state, and the abatement of fluorescent intensity commencing with dimer formation could not be explained. From this lower state, which is metastable owing to the prohibition of conversion, the molecule finally returns to the stationary state by additional non-radiative processes.

[illegible],
the fluorescent spectrum in Fig 9 should [illegible]
interval of 1,[illegible] cm^{-1} from the [illegible]
similar relation in its intensity to the monomeric band as that existing between the corresponding absorption b[ands]. Since the monomeric fluorescence band in aqueous solution has its maximum at [illegible],[illegible]50 cm^{-1}, this band ought to be observable at 17,[illegible]00 cm^{-1}. This is not the case as shown by spectra up to concentrations of $1 \cdot 10^{-4}$ molar. With the exception of concentration-dependent displacements, the curve maintains a constant configuration and progresses in the manner [illegible] in connection with steadily decreasing monomeric concentration. While certain indications of such a band do exist at a concentration of $1 \cdot 10^{-3}$ molar, this may not be considered a gross transgression of the prohibition of emission, since the intensities in the chart are relative, and the absolute intensity of the red band in comparison to the green band is very low. Quantitative measurements of differences in intensity inherent in the various fluorescent bands have been started; results will be published in a subsequent paper.

Assuming further symmetrical combination of dimers to more highly aggregated associations, e.g. tetramers, hexamers, octamers, etc., in which the planar molecules are superimposed in the manner by which dimers were formed out of monomers, and the ring N atoms are alternately opposed with their charges, a logical continuation of Förster's thought leads to division into a correspondingly large number of terms upon light excitation of the higher molecule, where the upper terms are permitted conversion, while the lower [illegible] in conformance with the rule of selection. In a [illegible]
[illegible]

ibility of such a conclusion and attributed the absorption band with the shortest waves to a highly polymeric, loose association with a structure of similar symmetry. Provided the persistence of the molecule in the metastable state could be extended under particularly favorable conditions, an emission from these lower states might not be a short-lived fluorescence, but a phosphorescence observable in the afterglow.

With increasing concentrations, the absorption spectra indeed show a continuous change in the position of the band center toward higher frequencies. This displacement must be considered a superimposition of different short wave bands of relatively close groupment with a continually changing intensity. The division into terms is certainly trivial due to the weak linkage of the individual electron oscillators, resulting in a gradually abating absorption without signs of phasing. It seems important in this connection that a curve intersection appears on the long wave side, which indicates an increasing, concentration-dependent intensity of long-waved, very weak absorption bands, probably attributable to poor convertibility in these metastable states. This becomes clear under the assumption that the associative orientation is not ideal at room temperature and that the compensation of the electric moment in the case of unsymmetrical oscillation is not zero, but a small finite quality.

Fluorescence spectra in the concentration range $1 \cdot 10^{-3}$ to $1 \cdot 10^{-4}$ molar distinctly show the energy levels to be expected from the expanded theory; their positions may be approximated at 17,500; 16,600; 15,800; 15,250 and 14,700 cm^{-1}. Since the levels are in a distinct sequence with rising concentrations, they may be considered proof of difined, reversible phases of association. In the meantime, this observation has been confirmed by measurements in organic solvents at low temperatures. The afterglow

to be expected from the metastability of these states cannot be demonstrated visually in aqueous solutions, since the excited molecules are rapidly deactivated by thermal impacts following excitation or fluorescence.

Fig 11 represents the material derived to date (11) from spectroscopic data in the form of a term diagram for the monomer and higher associations. In order to permit relative comparison, the electron ground state has been equalized; in absolute considerations, this level drops constantly during association due to the energy gain. The precise value for dimers was established at 5.7 kcal and should be compounded by a similar quantity for higher associations. The arrows drawn in Fig 11 point up to the permissible absorption conversions, down for the return through fluorescence to the electron ground state; the thickness of line indicates the probability of conversion and the broken lines the non-emission conversions by means of impact or internal molecular oscillation of nuclei. Between the ground state and the first level of excitation there are the "metastable" energy states resulting from the structural course of fluorescent intensity, entered according to their probability and energetic position. This determination is again relative, for the metastable energy levels may have small intervals and the electron ground state may be further divided by overlapping nuclear oscillating levels, as in the case of monomers. Such a representation makes intelligible the distinct gradation of bands observed in fluorescence, which were not seen in absorption due to the close proximity of the upper states.

It should be mentioned in closing that Strugger's assumption of a reversible, concentration-dependent polymerization of the acridine orange

dye action is essentially correct. The high polymers with strong chromophoric linkage found by Scheibe in the pseudoisocyanines could not be demonstrated in this case.